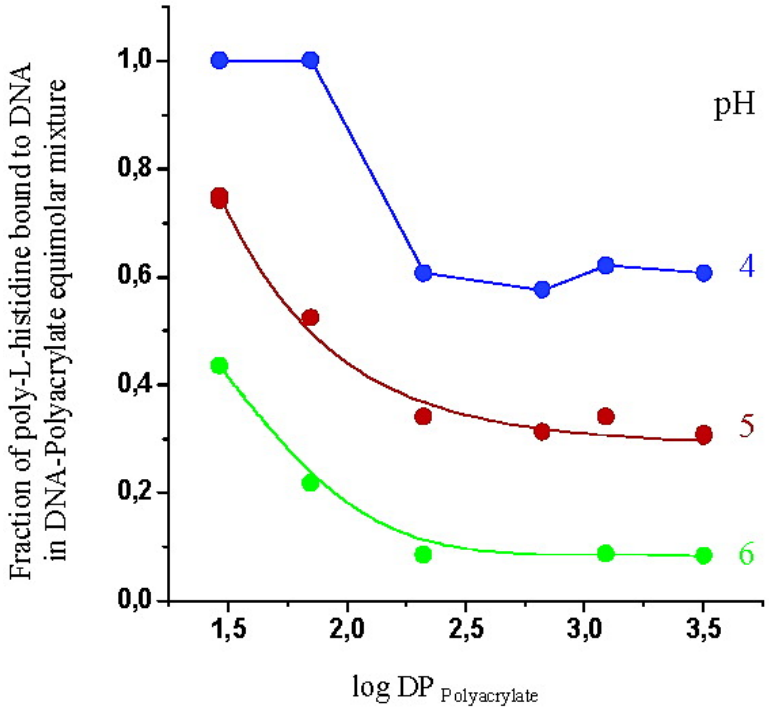
Article

Competitive Reactions in Solutions of Poly-l-histidine, Calf Thymus DNA, and Synthetic Polyanions: Determining the Binding Constants of Polyelectrolytes

Alexander N. Zelikin, Elizabeth S. Trukhanova, David Putnam, Vladimir A. Izumrudov, and Andrey A. Litmanovich

J. Am. Chem. Soc., **2003**, 125 (45), 13693-13699• DOI: 10.1021/ja036387y • Publication Date (Web): 21 October 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 8 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

J|A|C|S
ARTICLES
Published on Web 10/21/2003

Competitive Reactions in Solutions of Poly-L-histidine, Calf Thymus DNA, and Synthetic Polyanions: Determining the Binding Constants of Polyelectrolytes

Alexander N. Zelikin,[†] Elizabeth S. Trukhanova,[†] David Putnam,[‡] Vladimir A. Izumrudov,[†] and Andrey A. Litmanovich*[,§]

Contribution from the Department of Chemistry, M.V. Lomonosov Moscow State University, 119992 Moscow, Russia, School of Chemical and Biomolecular Engineering, and the Biomedical Engineering Program, Cornell University, 270 Olin Hall, Ithaca, 14853 New York, USA, and Moscow State Automobile & Road Technical University, 64 Leningradsky Prospekt, 125829 Moscow, Russia

Received May 28, 2003; E-mail: lit@chem.madi.ru

Abstract: The physicochemical characteristics of a nonviral gene delivery system will govern its functional bioactivity; however, empiricism dominates the literature in this field, and a significant deficiency of quantitative investigation and evaluation of nonviral gene delivery vehicles remains. Herein, we derive a physical model and experimental method to quantitatively determine the binding constants between a model polycationic nonviral gene delivery vehicle poly-L-histidine (PLH) and calf thymus DNA. The approach has utility to a variety of systems and is not limited to the described polymer model. The interaction of PLH with DNA was monitored by fluorescence quenching of an ethidium bromide probe in the pH range 4 to 8. The interaction increased with pH decrease with the most pronounced change between pH 6 and 7. The obtained pH-dependence of fraction of salt bonds formed between PLH and DNA was used to estimate pK_a of PLH in the presence of DNA, which equaled 6.24. The interaction of PLH with DNA in the presence of added synthetic polyanions was studied by the same approach and found to be controlled by pH, nature of the charge groups of the polyanion, and its degree of polymerization. In the mixture with sodium poly(styrenesulfonate) the interaction was negligible in the whole studied pH range, whereas in the mixtures with sodium poly(acrylate) (PA) or sodium poly(methacrylate), DNA was able to compete effectively for the binding with PLH. For PA samples with degree of polymerization higher than degree of polymerization of PLH, $DP_{PA} > DP_{PLH}$, the fraction of polycation bound to DNA was constant regardless of DP_{PA}. In contrast, at $DP_{PA} < DP_{PLH}$, a pronounced increase in the bound fraction was observed. It substantiates the notion that the binding energy of two polymers is mainly controlled by the DP of the shorter component of polyelectrolyte complex. The data on PLH distribution between DNA and added polyanion with different values of DP were treated according to the developed procedure to yield the effective binding constants of PLH with DNA and polyanion-competitor, calculated both per mole of interacting units K_1 and mole of interacting chains K_n. In all cases, K_1 had similar numerical values reflecting common type of interaction stabilizing the complexes, i.e., electrostatics. Slight variation of K_1 yielded in drastic changes in K_n and alteration of dominance of PLH interaction with DNA or synthetic polyanion. The results of the study can have a high impact in deriving the correlation between the binding constant of a polycation to DNA and its ability to serve as gene delivery vehicle.

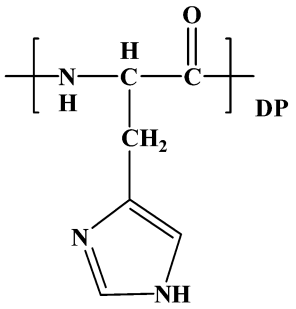
Introduction

The successful delivery of therapeutic DNA to cells offers the possibility of treating diseases at the genetic level. DNA delivery may be propagated by both viral (i.e., adenovirus, retrovirus) and synthetic (i.e., polycations and cationic lipids) vectors.[1] DNA delivery via polycations is strongly dependent upon the polycation's macromolecular composition and characteristics, e.g., chain length, charge density, etc.[1−3] The data on the influence of a polycation's characteristics on its ability to serve as gene delivery vehicle can be used as basis for the creation of physicochemical models capable of predicting the efficiency of delivery performed by different polycations.[4−6]

[†] Department of Chemistry, M.V. Lomonosov Moscow State University.
[‡] School of Chemical and Biomolecular Engineering, and the Biomedical Engineering Program, Cornell University.
[§] Moscow State Automobile & Road Technical University.

(1) Kabanov, A. V.; Felgner, P. L.; Seymour, L. W. *Self-Assembling Complexes For Gene Delivery: From Laboratory To Clinical Trial*; John Wiley and Sons: New York, 1998.
(2) (a) Luo, D.; Saltzman, W. M. *Nat. Biotechnol.* **2000**, *18*, 33. (b) Godbey, W. T.; Wu, K. K.; Mikos, A. G. *J. Biomed. Mater. Res.* **1999**, *45*, 268;
(3) Wolfert, M. A.; Dash, P. R.; Nazarova, O.; Oupicky, D.; Seymour, L. W.; Smart, S.; Strhalm, J.; Ulbrich, K. *Bioconjugate Chem.* **1999**, *10*, 993.
(4) Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. *Biotechnol. Bioeng.* **2000**, *67*, 598.
(5) Zelikin A. N.; Putnam, D.; Shastri, P.; Langer, R.; Izumrudov, V. A. *Bioconjugate Chem.* **2002**, *13*, 548.
(6) Reineke, T. M.; Davis, M. E. *Bioconjugate Chem.* **2003**, *14*, 255.

Scheme 1



Yet, in the majority of cases, the study of the DNA−polycation interaction is descriptive and yields little if any quantitative information, such as binding constants *K*, energy of complex formation, etc. In part, this is due to a pronounced cooperative mode of electrostatic interaction between DNA and polycations that is characterized by *K* values too high to be established by common techniques (subsequent dilution etc.). For example, the binding constant for an oligolysine with only 8 charged groups in the chain to poly(dT) is $\sim 10^4-10^6$ M^{-1} depending on the chain length of the latter.[7] In turn, high *K* values result from the dramatic increase in entropy of the system that stems from the liberation of counterions from the ionic atmospheres of the polyelectrolytes and which is the driving force of complex formation between a highly charged polyanion and polycation.[8] Recently, it was shown that excessively high affinity of a polycation to DNA can be a limiting step of transfection due to limited separation of the DNA from the gene carrier.[4] The liberation of DNA is thought to proceed via competitive interaction of the DNA−polycation complex with charged intracellular components,[9] yet quantitative proof of this hypothesis is lacking.

Herein, we report the investigation of selective binding of a polycation to DNA and a synthetic polyanion-competitor to model the separation of DNA from a polycation in the living cell. Additional insight into the electrostatic interplay is provided through a theoretical treatment of the data. The polycation used was poly-L-histidine (PLH, Scheme 1). Utilization of this cationic polyelectrolyte as well as other imidazole-containing polymers is intriguing due to its ability to induce the so-called proton sponge mechanism of the endosomal escape of the gene delivery vehicle.[10] An attractive feature of this polymer is its relatively low intrinsic pK_a ($\sim$5−6), which offers the means to control the interaction of PLH with DNA through the variation of the pH in the narrow range close to physiological values.

Experimental Part

A. Reagents. Ethidium bromide (EB) was purchased from Sigma (USA). Concentration of EB in solutions was determined spectroscopically assuming a molar extinction coefficient $\epsilon_{480} = 5600$ L mol^{-1} cm^{-1} at 480 nm.[11] In all experiments, twice distilled and additionally purified by Milli-Q (Millipore, USA) water was used. NaCl and Tris, PBS, MES, and HEPES buffers were purchased from Sigma (St. Louis, USA).

B. Polymers. 1. Poly-L-histidine Hydrochloride. Poly-L-histidine hydrochloride (PLH) sample with DP = 91 was purchased from Sigma (St. Louis, USA) and used without further purification.

2. Calf Thymus DNA. The sodium salt of highly polymerized calf thymus DNA ($\sim$10,000 base pairs) was purchased from Sigma (St. Louis, USA) and used without further purification. The purity of the DNA samples was verified by UV−vis spectroscopy and thermal denaturation profile of the sample. Concentration of DNA phosphate groups was determined by UV absorbance measurements at 260 nm assuming molar extinction coefficient $\epsilon_{260} = 6500$ L mol^{-1} cm^{-1}.[12]

3. Poly(sodium styrene sulfonate). Poly(sodium styrene sulfonate) (PSS) standard monodisperse sample with number-average degree of polymerization (DP) 20 was purchased from Pressure Chemicals (Pittsburgh, PA) through Serva (Germany); PSS sample with DP = 500 was purchased from Polysciences (Warrington, PA).

4. Poly(acrylic acid). Poly(acrylic acid) (PA) samples with weight average degree of polymerization DP = 210, 660, 1200, and 3200 were synthesized, fractionated and characterized as described elsewhere.[13] The polydispersity of the samples was M_w/M_n 1.25 ± 0.05. The samples with DP = 30 and 70 were purchased from Aldrich (USA) and used without purification.

5. Poly(methacrylic acid). Poly(methacrylic acid) (PMA) sample with weight average degree of polymerization DP = 1800 was synthesized from methacrylic acid according to the procedure analogous to that for PA.[13]

C. Methods. 1. Spectrophotometric Measurements. Spectrophotometric measurements were performed using a Hitachi 150−20 Spectrometer (Japan) in a water-thermostatic cell under constant stirring.

2. Fluorescence Intensity. Fluorescence intensity was measured using Jobin-Yvon-3CS Spectrofluorometer (France) with a water-thermostatic stirred cell holder. The measurements were performed in a quartz fluorescence cell with continuous stirring. The excitation and emission wavelengths were 535 and 595 nm, respectively. In all experiments, DNA·EB complex of composition [P]/[EB] = 4/1, where [P] and [EB] are molar concentrations of the phosphate groups and the dye, respectively, were used to allow the maximal possible fluorescent signal.[14] Solutions of polyelectrolyte complexes were prepared directly in the quartz cell by mixing of definite volumes of stock solutions of the polymers.

All measurements were performed at 25 °C, the initial concentration of DNA in the cell was 4×10^{-5} M. Preliminary experiments showed that the thermodynamic equilibrium in each of the systems described below was achieved in 5−10 min in the presence of 0.05 M NaCl. Thus, all buffer solutions were charged with 0.05 M NaCl, and 10 min time intervals separated the additions of titrant portions. Several runs were performed for each experiment and the results were reproducible within 5% experimental error.

Results and Discussion

The interaction of poly-L-histidine (PLH) with calf thymus DNA was monitored by the fluorescence quenching technique with the use of the cationic dye ethidium bromide (EB). Fluorescence of this dye in aqueous media is negligible, whereas in the presence of native double stranded DNA, fluorescence ignition occurs due to intercalation of the dye between base pairs and formation of DNA·EB complexes. This feature of EB is widely utilized to visualize the migration of DNA in gel electrophoresis experiments. Addition of polycations with different structural characteristics to an aqueous solution of DNA·EB complex results in a pronounced decrease in fluores-

(7) Zhang, W.; Ni, H.; Capp, M. W.; Anderson, C. F.; Lohman, T. M.; Record, M. T. *Biophys. J.* **1999**, *76*, 1008.
(8) Kabanov, V. A. In *Macromolecular Complexes in Chemistry and Biology*; Dubin, P. L., Ed.; Springer-Verlag: Berlin, and Heidelberg, Germany, 1994; p.151.
(9) Erbacher, P.; Roche, A. C.; Monsigny, M.; Midoux, P. *Bioconjugate Chem.* **1995**, *6*, 401.
(10) Behr, J.-P. *Chimia* **1997**, *51*, 34.
(11) Warring, M. J. *J. Mol. Biol.* **1965**, *13*, 269.
(12) Olins, D. E.; Olins, A. L.; von Hippel, P. H. *J. Mol. Biol.* **1967**, *24*, 157.
(13) Kharenko, O. A.; Kharenko, A. V.; Kalyuzhnaya, R. I.; Izumrudov, V. A.; Kasaikin, V. A.; Zezin, A. B.; Kabanov, V. A. *Vysokomol. Soedin.* **1979**, *A XXI*, 2719.
(14) Stratling, W. H.; Seidel, I. *Biochemistry* **1976**, *15*, 4803.

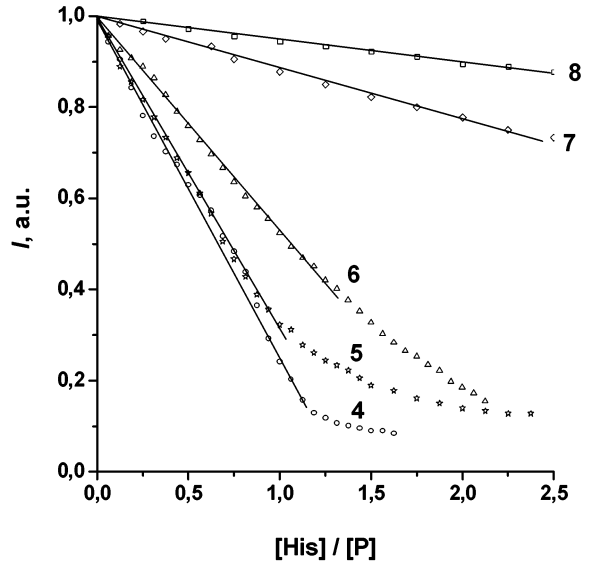


Figure 1. Fluorescence intensity I of the mixtures of DNA with PLH as a function of ratio of PLH imidazole and DNA phosphate groups concentrations at different pH (marked at each titration curve). [P] = 4×10^{-5}M; [P]/[EB] = 4/1; 25 °C.

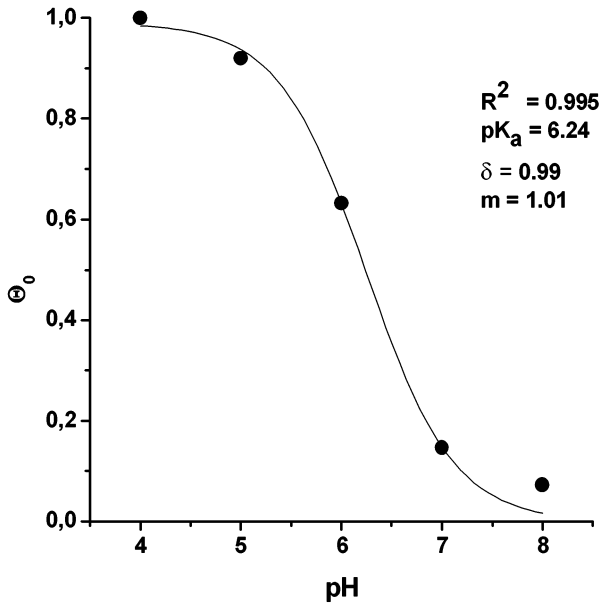


Figure 2. pH-Dependence of the fraction of salt bonds Θ_0 formed between PLH and DNA. Experimental conditions are the same as in the legend of Figure 1.

cence intensity due to competitive displacement of the dye by the formation of a polycation polyelectrolyte complex with DNA.[15]

Fluorimetric titration of DNA·EB aqueous solutions having fluorescence intensity I_0 with PLH solutions was conducted at different pH. The data are presented in Figure 1 as a dependence of relative fluorescence intensity I/I_0 of the solution as a function of the ratio $\varphi \equiv$ [His]/[P], where [His] and [P] are the concentrations of monomer units of PLH and phosphate groups of DNA, respectively. The decrease in I/I_0 is observed in all mixtures which implies formation of DNA−PLH polyelectrolyte complex at all studied pH values. The lowering of pH resulted in more pronounced quenching with the maximal effect at pH 4. In the latter case, the complete fluorescence quenching occurred at ratio close to stoichiometric, $\varphi \approx 1$. This finding suggests that at pH 4 almost all histidine units of PLH are charged and involved in formation of ion pairs with phosphate groups of DNA. In other words, at this pH the degree of conversion of polyelectrolyte reaction θ_0 defined as the ratio of a current number of salt bonds formed between DNA and PLH to the maximal possible value, i.e., the degree of polymerization of the shorter component of the complex −PLH, is close to unity.

In all mixtures up to $\varphi \cong 1$, the dependence of the fluorescence intensity I/I_0 is a linear function of the ratio of the components, φ (Figure 1). Therefore at a fixed φ the quenching of fluorescence at pH_{exp}, $(I_0 - I)_{pH\ exp}$, normalized to the quenching at pH 4, $(I_0 - I)_{pH\ 4}$, is the measure of θ_0 formed at this pH[16]

$$\Theta_0 = \frac{(I_0 - I_{exp})_{pH\ exp}}{(I_0 - I_{exp})_{pH4}} \qquad (1)$$

According to this, the θ_0 values were calculated from results presented in Figure 1 and plotted as a function of pH (Figure 2). The increase in pH from 4 to 5 resulted in a minor change of Θ_0. Further increase in pH significantly disrupted the system of the salt bonds (reflected by the drop of Θ_0), and at pH 8 the electrostatic interaction of PLH with DNA became negligible. According to these data, the interaction between PLH and DNA can be tailored via the change in pH in a narrow range close to the physiological values.

The ionization of a polybase in solution in the absence of oppositely charged polyelectrolytes can be expressed via pH of the medium and the pK_a value of its conjugate acid according to the following equation[17]

$$pH = pK_a - m \times \log \frac{\alpha}{1 - \alpha} \qquad (2)$$

where α is the degree of ionization. The multiplier m is the interaction parameter, which reflects the fact that ionization of the neighboring groups on the polyelectrolyte chain requires overcoming the electrostatic interaction of the neighbor ions. While for monomeric acids and bases $m = 1$; for weak polyelectrolytes in their salt-free solution m equals ~2.[18]

As all the ionized groups of a polyelectrolyte taken in lower content are involved in the formation of salt bonds,[19] for the system under study $\alpha \equiv \Theta_0$; to take into account the possible inaccuracy of the assumption that at pH 4 all imidazole groups are charged, eq 2 was modified into

$$pH = pK_a - m \times \log \frac{\Theta_0}{\delta - \Theta_0} \qquad (3)$$

where δ is the ratio of Θ_0 values at pH 4 and the maximal possible value, $\delta \leq 1$. The iterative fitting of the experimental

(15) Izumrudov, V. A.; Zhiryakova, M. V.; Kudaibergenov, S. E. *Biopolymers* **1999**, *52*, 94.

(16) (a)Izumrudov, V. A.; Zhiryakova, M. V.; Akritskaya, N. I. Fluorescence Quenching Technique for Study of DNA-containing Polyelectrolyte Complexes. In *Advanced Macromolecular and Supramolecular Materials and Processes*; Geckeler, K., Ed.; Kluwer Academic/Plenum Publishers: Norwell, MA, 2003; p 277. (b) Erbacher, P.; Roche, A. C.; Monsigny, M.; Midoux, P. *Biochim Biophys Acta* **1997**, *1324*, 27.

(17) Katchalsky, A., Spitnik, P. *J. Polym. Sci.* **1947**, *2*, 432.

(18) Tsuchida, E.; Osada, Y.; Abe, K. *Makromol. Chem.* **1974**, *175*, 583.

data points according to eq 3 with variation of pK_a of imidazolium ion and δ and m values was carried out and the best fit is presented in Figure 2 (the line connecting the data points). Both δ and m values equaled unity suggesting that at pH 4 the fraction of salt bonds formed is unity and that the presence of the oppositely charged polyion (DNA) eliminates the electrostatic hindrance of ionization of neighboring imidazole groups on PLH. The resulting pK_a value equaled 6.24, whereas the pK_a of PLH in solution in absence of added polyanion is ~5.75.[20] Thus, the presence of a strong polyanion eases the ionization of imidazole groups of PLH resulting in a shift of pK_a of its conjugate acid to higher values. Such behavior is characteristic for weak polyelectrolytes, yet in this particular case the shift in pK value is only moderate, ~0.5 pH units compared to more than 2 pH units for polycarboxylic acids in the presence of strong polycations.[18] This difference can be attributed to possible hydrogen bonding between PLH and DNA.[21] In spite of the fact that the presence of DNA induces protonation of the bound PLH, formation of hydrogen bonds between DNA bases and uncharged imidazole groups can hinder their protonation.

As mentioned previously, PLH and imidazole-containing polymers in general are attractive polycations for gene delivery. It is known that one of the potential barriers in transfection is the liberation of the reporter gene from its complex with a polycation-carrier.[4] Because in the majority of cases, DNA−polycation complexes are tolerant to physiological ionic strength, it was hypothesized that the release of nucleic material from the carrier proceeds via a competitive reaction between the components of the gene delivery vehicle (including DNA) with charged intracellular species such as sulfonated sugars, membrane components, etc.[9] Yet quantitative data on such process are scarce. In the current work, we aimed to assess the binding constants of PLH with various polyanions and thus verify this hypothesis on DNA−PLH complexes in mixtures thereof with negatively charged polyelectrolytes.

The first polyanion studied as a competitor to DNA for binding with PLH was poly(styrenesulfonate) (PSS) anion. Macromolecules with sulfonate and/or sulfate groups such as, poly(vinylsulfonate),[22] poly(vinyl sulfate),[23] and PSS[24] are known to be very strong competitors to other polyanions for binding with polycations. Indeed, introduction of PLH into a solution containing equimolar concentrations of DNA and PSS (DP = 500) at pH 5 resulted in a minor change of the fluorescence up to $\varphi = 1$ (Figure 3, curve 2) which suggests that PLH practically does not interact with DNA in the presence of PSS. Further addition of PLH was followed by a decrease of the fluorescence analogous to that observed on titration of DNA with PLH (Figure 3, curve 1). Because the initial concentration of sulfonate groups and phosphate groups in the mixtures was

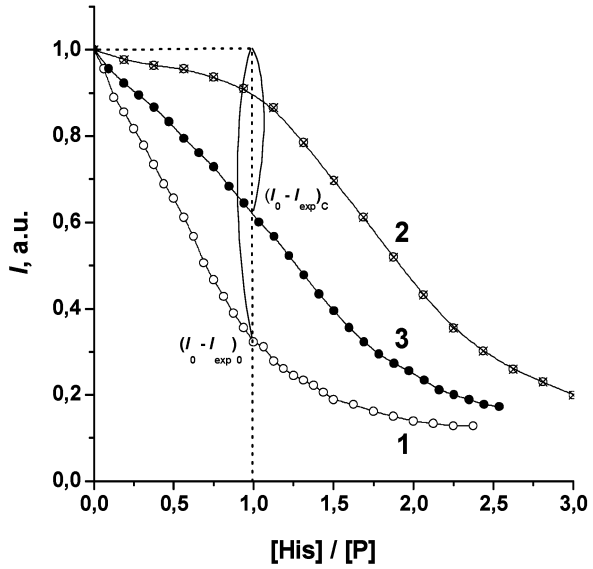


Figure 3. Dependence of fluorescence intensity of a PLH solution with DNA (1), equimolar mixture of DNA and PSS (2), and equimolar mixture of DNA with PMA (3) on the ratio of concentrations of PLH imidazole and DNA phosphate groups. Experimental conditions are the same as in the legend of Figure 1.

the same, these data imply that the interaction of PLH with DNA becomes possible only if all PSS chains already formed the complex with PLH. The same observation was true even for the oligomeric sample of PSS, DP = 20. Despite the relatively small amount of sulfonate groups per polyanion chain the presence of this oligoanion substantially suppressed the interaction of PLH with DNA. For both highly polymerized PSS and the oligomeric sample, the described trend was observed in the whole studied range of pH 4 to 6.

A completely different mode of distribution of PLH between DNA and a polyanion-competitor was observed in the mixture with poly(methacrylate) anion (Figure 3, curve 3). Introduction of PLH into a solution containing equimolar concentrations of DNA and PMA (DP = 1800) at pH 5 was followed by the quenching of fluorescence, yet the quenching was much less pronounced as compared to the system containing DNA with no competitor added. This observation suggests a certain distribution of PLH between DNA and PMA with a fraction of PLH bound to DNA greater than zero but lower than unity. This finding agrees well with the reported ability of polycarboxylic acids to compete with DNA for binding with polycation.[25] Thus, polyaspartic acid can displace DNA from complex with poly-L-lysine, yet unlike sulfonate containing polyanion in this case up to 10-fold excess of polyaspartic acid is required.[3,26]

At a fixed pH, the fraction Θ of the salt bonds formed between DNA and PLH in the presence of an added competitor can be calculated from the linear slopes of the curves presented in Figure 3 as the ratio of quenching of fluorescence in the (DNA−PLH−polyanion) solution to that for DNA−PLH solution

$$\Theta = \frac{(I_0 - I_{exp})_C}{(I_0 - I_{exp})_0} \qquad (4)$$

Note that unlike in eq 1, the values of fluorescence intensity utilized in eq 4 for the systems with and without the added

(19) Dautzenberg, H. Polyelectrolyte Complex Formation in Highly Aggregating Systems: Methodical Aspects and General Tendencies. In *Physical Chemistry of Polyelectrolytes;* Radeva, T., Ed.; Marcel Dekker: New York, Basel, 2001; p 743.
(20) Patchornik, A.; Berger, A.; Katchalski, E. *J. Am. Chem. Soc.* **1957**, *79*, 5227.
(21) Burckhardt, G.; Zimmer, Ch.; Luck, G. *Nucleic Acids Res.* **1976**, *3*, 561.
(22) Katayose, S.; Kataoka, K. *J. Pharm. Sci.* **1998**, *87*, 160.
(23) Kakizawa, Y.; Harada, A.; Kataoka, K. *Biomacromolecules* **2001**, *2*, 491.
(24) (a) Izumrudov, V. A.; Bronich, T. K.; Zezin, A. B.; Kabanov, V. A. *J. Polym. Sci., Polym. Lett. Ed.* **1985**, *23*, 439. (b) Kabanov, V. A.; Zezin, A. B.; Izumrudov, V. A.; Bronich, T. K.; Bakeev, K. N. *Makromol. Chem., Suppl.* **1985**, *13*, 137. (c) Oupicky, D.; Konak, C.; Dash, P. R.; Seymour, L. W.; Ulbrich, K. *Bioconjugate Chem.* **1999**, *10*, 764.

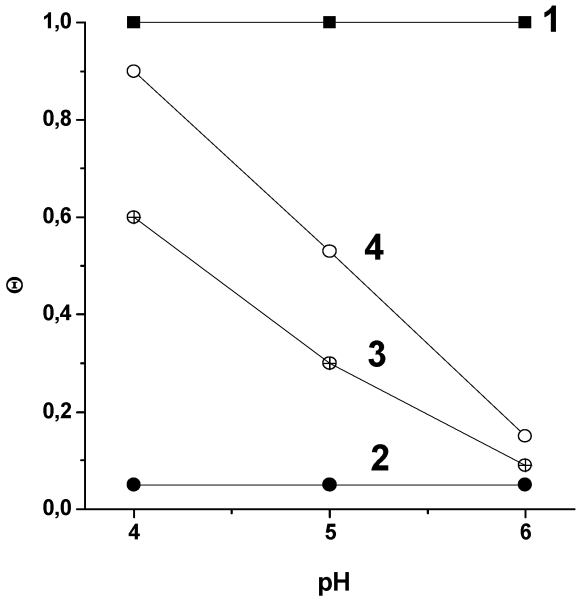


Figure 4. pH-Dependence of the fraction of salt bonds Θ formed by PLH and DNA in the mixture of PLH with DNA (1), equimolar mixture of DNA and PSS (2), PA (3) and PMA (4).

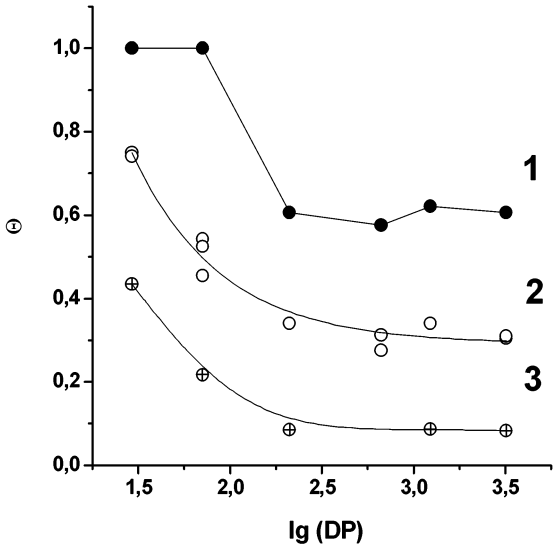


Figure 5. Dependence of fraction of salt bonds formed by PLH and DNA in solutions of PLH and an equimolar mixture of DNA and PA at pH 4 (1), 5 (2), and 6 (3) on the logarithm of PA degree of polymerization.

polyanion-competitor are measured at the same pH. Θ-pH profiles obtained according to eq 4 from the titration curves of solutions of DNA mixtures with highly polymerized PSS or polycarboxylic acids with solution of PLH are depicted in Figure 4. Obviously, in the absence of the competitor Θ identically equals unity in the whole pH range (curve 1). In the DNA−PSS mixtures, Θ value was low at all pH and did not exceed 0.05 (curve 2). As mentioned above, these data imply that the added PLH predominantly interacts with the sulfonate baring polyanion and the amount of imidazole groups electrostatically bound to DNA does not exceed 5%.

In contrast to fully charged DNA and PSS, the ionization of polycarboxylic acids in acidic media is diminished. Nevertheless, highly polymerized poly(acrylic acid) (PA) proved to be sufficiently strong competitor to DNA binding ca. 40% of PLH at pH 4 (Figure 4, curve 3). Increase in pH is followed by a drastic decrease in Θ, and at pH 6 this polyanion is as strong competitor to DNA as PSS. This result is readily explained by the growing ionization of carboxylic moieties of PA from pH 4 to 6. The substitution of PA for poly(methacrylic acid) results in substantial shift of PLH distribution between DNA and an added competitor (Figure 4, curve 4). Thus, while at pH 4, the value of Θ in the PA-containing system equaled 0.6, in the case of PMA, Θ equaled 0.9 indicating predominant interaction of the polycation with nucleic acid at this pH. Similar to the case of PA as a competitor, the increase in pH is followed by a Θ decrease, yet in the PMA-containing system, the Θ value is higher at any pH in the whole studied pH range. The latter can be attributed to markedly higher pK_a value of PMA.[18] Thus, for low ionic strength conditions, a pK_a of about 6 to 7 is usually reported for PMA, while a lower pK_a ranging from 5 to 6 is usually found for PA.

To investigate the influence of molecular weight of polyanion on its ability to compete with DNA for binding with PLH, samples of poly(acrylic acid) with DP in the range 30 to 3200 were used. The results of these experiments are presented in Figure 5 as dependencies of Θ on logDP of the polyacid that were obtained at pH 4 (curve 1), pH 5 (curve 2), and at pH 6 (curve 3). As discussed above, the increase in pH leads to substantial re-distribution of PLH toward binding with PA; this observation is true in the whole studied range of DP of the polyacid. Note, that DP decrease from 3200 to 210 scarcely affects the degree of conversion, whereas further shortening of the polyacid results in a drastic increase in a number of PLH groups bound to DNA. Thus, at pH 6 highly polymerized PA binds predominantly with the polycation, while distribution of PLH between oligomeric samples of PA and DNA at this pH becomes almost even (curve 3). The analogous trend is observed at pH 5 and 4. Moreover, in the latter case (curve 1) PA samples with DP 30 and 70 are not able to bind any PLH in the presence of DNA. In other words, at pH 4 short PA chains with DP values lower than DP = 91 of PLH cannot compete with DNA for the binding. This finding substantiates the notion that the binding energy of two polymers is mainly controlled by the degree of polymerization of the shorter component of polyelectrolyte complex, in our case, the shorter component in each of the complexes formed.[8,27]

The adsorption of an oligomer with degree of polymerization DP = n onto a one-dimensional lattice (polymer) can be expressed with an isotherm[27]

$$\frac{1}{c}\frac{\beta}{1-\beta}\exp\left\{\frac{\beta}{1-\beta}\right\} = \exp\left\{\frac{-n\Delta G_1^{\,0}}{RT}\right\} \qquad (5)$$

where β is the share degree of filling of the polymer groups with the oligomer (fraction of monomer units of the polymer occupied by the oligomer), c −concentration of the free oligomer, $\Delta G_1^{\,0}$ is the free energy of interchain bond formation calculated per mole of monomer units of the oligomer. The right-hand side of eq 5 is the equilibrium constant of adsorption, i.e.,

(25) Izumrudov, V. A.; Kargov, S. I.; Zhiryakova, M. V.; Zezin, A. B.; Kabanov, V. A. *Biopolymers* **1995**, *35*, 523.
(26) Katayose, S.; Kataoka, K. *Bioconjugate Chem.* **1997**, *8*, 702−707.
(27) Papisov, I. M.; Litmanovich, A. A. *Adv. Polym. Sci.* **1989**, *90*, 139.

the binding constant of the oligomer K_n with DP = n

$$K_n = K_1^{\,n} \tag{6}$$

where K_1 is the binding constant per single bond formed

$$K_1 = \exp\left\{\frac{-\Delta G_1^{\,0}}{RT}\right\} \tag{7}$$

In the mixture of two lattices, P_1 and P_2, and an oligomer with DP of the latter not exceeding the value of this parameter for each of the lattices, eqs 5−7 are applicable to the interaction of the oligomer with each of the polymers. If the number of bonds formed by the oligomer with each of the polymers is the same and equals n, then the selectivity of oligomer binding to P_1 and P_2 in their equimolar mixture can be written as

$$\psi = \lim_{(\beta_1+\beta_2)\to 0}\left(\frac{\beta_1}{\beta_2}\right) = \left(\frac{K_{11}}{K_{12}}\right)^n \tag{8}$$

where $K_{1(i)}$ is the binding constant of the oligomer to P_i calculated per mole of binding units. As experimentally it is impractical to meet the ($\beta_1 + \beta_2 \to 0$) requirement in further calculations the following equation was utilized

$$\psi = \frac{B_1}{B_2} = \left(\frac{K_{11}}{K_{12}}\right)^n \tag{9}$$

where

$$B_i = \frac{\beta_i}{1-\beta_i}\exp\left(\frac{\beta_i}{1-\beta_i}\right) \tag{10}$$

which reduces to eq 8 under ($\beta_1 + \beta_2 \to 0$) conditions.

Assigning DNA as polymer P_1, polyanion-competitor as polymer P_2 and PLH as an oligomer, n can be calculated according to

$$n = DP_{\mathrm{PLH}} \times \Theta_0 \tag{11}$$

We further need to assume that share degree of filling of a polymer with the oligomer identically equals the fraction of salt bonds formed between this polymer and oligomer, i.e., the distribution of the PLH chains can be expressed via the distribution of the salt bonds formed. Thus, for DNA $\beta_1 = \Theta$, for the polyanion-competitor $\beta_2 = (1 - \Theta)$, and eq 9 can be transformed into

$$\left(\frac{K_{11}}{K_{12}}\right)^n = \frac{\Theta^2}{(1-\Theta)^2}\exp\left[\frac{\Theta^2-(1-\Theta)^2}{\Theta\times(1-\Theta)}\right] \tag{12}$$

Thus, experimental Θ_0 and Θ values can be utilized to calculate the ratio of the binding constants of PLH to DNA and a polyanion-competitor according to eq 12 with a prerequisite that n value has a constant value for the two pairs of interacting polyelectrolytes. This condition can be met in the case of long chains of polyanions, in particular for the data presented in Figure 4. The experimental Θ values and calculated values of ψ and K_{11}/K_{12} are listed in Table 1. The value of ψ gives essentially the same information as the corresponding Θ value. At $\psi > 1$ ($\Theta > 0.5$), binding of PLH with DNA is more favorable than with the added polyanion-competitor, whereas at $\psi < 1$ ($\Theta < 0.5$) the distribution of PLH is shifted toward interaction with the synthetic competitor.

Table 1. Θ, ψ and K_{11}/K_{12} Values for DNA−PLH−Polyanion-Competitor Interaction at Different pH

	pH	Θ	ψ	K_{11}/K_{12}
PA	4	0.6	5.7	1.02
	5	0.3	2.7×10^{-2}	0.96
	6	0.09	4.4×10^{-7}	0.76
PMA	4	0.9	5.9×10^{5}	1.16
	5	0.53	1.6	1.01
	6	0.15	1.3×10^{-4}	0.86
PSS	4	<0.05		0.5−0.75
	5	<0.05		0.5−0.75
	6	<0.05		0.5−0.75

The most important result of the calculations is the relative values of binding constants of PLH with the polyanions. As seen from the data of Table 1, even for the strongest competitor PSS, the ratio K_{11}/K_{12} is about 0.75, and in all other cases, this parameter is close to unity. It is noteworthy that a modest variation in the ratio of binding constants could change the Θ value from zero to unity. In other words**,** a rather small difference in the binding energy per pair of reacting units can yield a dramatic shift of the equilibrium of polycation distribution between the two polyanions. It needs to be emphasized that in these calculations, only the charged groups of PLH are taken into account, and for each of the polyanions, the binding constants are average of all the groups on their chains. Although for DNA and PSS, the ionization of the polyelectrolytes is complete and invariable of pH in the range 4−6, this is certainly not the case for the polycarboxylic acids. Accordingly, the K_1 value for PLH−DNA interaction is considered to be constant, whereas K_1 for PLH−PA or PLH−PMA is not. Thus, K_{11}/K_{12} value decreases with pH increase reflecting the undergoing ionization of PA (PMA).

Furthermore, our calculations demonstrate that data presented above can be used to obtain not only the relative values of binding constants of PLH with two polyanion-competitors but also the numerical value of each of the individual constants. In the case of $DP_{PA} > DP_{PLH}$, eq 5−12 are valid for this system as well as for mixtures of DNA and PSS. If the number of bonds formed by polyanion P_1 and polyanion P_2 with the present polycation is not the same, i.e., $n_1 \neq n_2$, then eq 9 is not applicable and is substituted with

$$\psi = \frac{K_{11}^{n_1}}{K_{12}^{n_2}} \tag{13}$$

$$\ln\psi = n_1 \ln K_{11} - n_2 \ln K_{12} \tag{14}$$

$$\ln\psi = \ln\left(\frac{K_{11}}{K_{12}}\right)^{n_1} + \ln K_{12}^{\,n_1\,-n_2} \tag{15}$$

$$\ln\psi = \ln\psi_0 + \ln K_{12}^{\,n_1-\,n_2} \tag{16}$$

where ψ_0 is the selectivity found in mixture of these polymers conditioned with $n_1 = n_2$, which can be taken from Table 1, n_1 is the DP of PLH and n_2 is DP of PA sample. Thus, the data of Figure 5 can be used to calculate the K_{12} value at different pH, the results of the calculations are listed in Table 2. These data, together with the data of Table 1 allow calculating the K_{1i} value

Table 2. Θ Values for DNA−PLH−PA Interaction and Calculated Therefrom K_1 Values for PLH−PA Interaction at Different pH

DP_{PA}	pH	Θ	K_1
30	5	0.75	1.15
30	6	0.44	1.68
70	5	0.50	1.32
70	6	0.22	1.63

Table 3. Effective Binding Constants K_1 for Interaction of PLH with Different Polyanions

	pH	K_1
PA	4	1.20
	5	1.25
	6	1.68
PMA	4	1.06
	5	1.22
	6	1.43
PSS		∼1.7 ÷ 2.5
DNA		1.26 ± 0.05

for PLH interaction with each polyanions, the results of the calculations are summarized in Table 3.

As mentioned above, the K_1 values for each of the polyanions−PLH interaction are binding constants averaged for all the polyanions' groups, not only the charged ones. Thus, the increase in K_1 with increase in pH in case of polycarboxylic acids is readily explained by the growing ionization of the carboxylic moieties hence stronger interaction with PLH. In the whole pH range, the K_1 value for PLH binding with PA is higher compared to its binding to PMA. As discussed previously, this result stems from the higher pK_a value of PMA compared to PA, hence the lower degree of ionization in pH range 4 to 6.

It needs to be clarified that the herein obtained K_1 values should be attributed only to the interaction of PLH with each of the polyions in the mixtures thereof hence are effective constants. Thus, eq 5 was utilized to describe the complex formation of the polycation with each of the two polyanions and further calculations were based on the result of the division of two isotherms and the energy of the unbound state of PLH is thought to be neglected. The binding constant of PLH to each of the polyanions in the absence of competitors therefore equals $\mathscr{K}_{1i}' = \mathscr{K}_{1i} \times A$, where A takes into account the PLH unbound state energy.

The developed approach can be used for studying competitive reactions in solutions of DNA and different negatively and positively charged (bio)macromolecules. In our recent experiments with ionene bromides as the polycations, the role of degree of polymerization and charge density of their chains in the competitive reactions became evident. The experiments to quantify the effects are underway. It is noteworthy that the K_n value for interaction of DNA with ionene-3,3 (DP = 220) in acidic media equaled ∼10^5, whereas the K_n value for PLH−DNA complex determined at the same pH was ca. 10^9 (Figure 6), i.e., almost 4 orders of magnitude higher. This finding agrees well with relatively low stability of DNA−ionene complex that dissociates completely at $C_{NaCl} \approx 0.6$M,[28] whereas the DNA−PLH complex remains stable even in 2 M sodium chloride solutions.

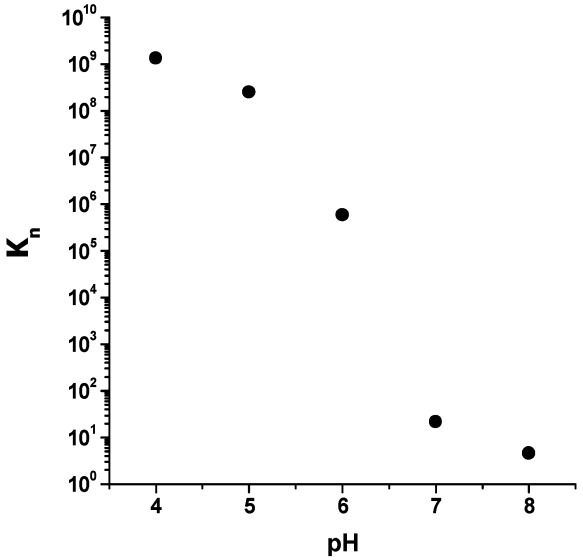


Figure 6. pH-Dependence of PLH−DNA binding constant calculated per mole of interacting chains.

Concluding Remarks

The results obtained strongly suggest that liberation of the DNA from the gene delivery vehicle can proceed via the competitive interaction of the components of the polyelectrolyte complex with other charged species. The herein calculated K values should be taken as effective binding constants, yet they adequately reflect the experimentally observed properties of polyelectrolyte complexes. The approach herein introduced to estimate the binding affinity of polyelectrolytes including DNA can be adopted to monitor the interaction of a variety of charged macromolecules and is not limited to the studied polyelectrolytes. The key potential application of the proposed approach is correlating the binding affinity of the carrier of genetic material to DNA and its utility as gene delivery vehicle; this is the subject of ongoing research.

JA036387Y

(28) Zelikin, A. N.; Litmanovich, A. A.; Paraschuk, V. V.; Sybatchin, A. V.; Izumrudov, V. A. *Macromolecules* **2003**, *36*, 2066.